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ANALYSIS OF THE VIRULENCE OF THE PLAGUE MICROBE AND PREPARATION OF LIVE PLAGUE VACCINES

Report 2. Study of the Cell Composition of Virulent Plague Strains of Different Origin

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The need to find new live plague vaccines is still urgent. Experience has shown that it is not always possible to prepare a good vacine from every plague strain. The reason is that the various strains differ in their ability to preserve their virulent and immunogenic properties. One of the authors (Malinina, manuscript) found that plague microbes stored for many years on nutrient media without subculturing lose their virulent and immunogenic properties more quickly than do strains isolated from gerbils. Osadchaya (1957), Punskiy (1957), and Levina (1960) state that gerbil strains of the plague microbe lose their virulence rather quickly when stored on nutrient media.

We endeavored to compare certain peculiarities in the loss of virulence by strains isolated from various carriers and to determine if there is a connection between the virulence of individual colonies in strains with their capacity to utilize rhamnose and glycerin. For this purpose we studied the cell composition of 4 virulent plague strains used in the "Mikrob" Institute as standards: 2 freshly isolated (363 and 380), used in the experiment several months after isolation, and 2 stored more than 10 years on nutrient media (231 and 293). Their properties are typical of representatives of the species, with pronounced virulence. Guinea pigs and white mice die from acute experimental plague when inoculated with 25 microbes obtained from the general population of the strains. Three of them belong to the continental variety (they ferment glycerin), 1 to the oceanic (it does not ferment glycerin). The characteristics of these strains are summarized in Table 1.

Table 1

Characteristics of the Plague Strains Investigated (General Population)

(1) Штамм	(2) Год выделения	(3) Очаг	(4) Вирулентность, м. т. (5) морские свинки	(6) белые мыши	(7) На 100 м. т. вырастают на средах (8) с 5% крови (9) Хигучи 28°	37°	28°	(10) Ферментация (11) рамнозы	(12) глицерина	(13) Лизис чумным фагом
231	1947	Сурчиный (14)	25	25	48	0	46	—	+	++
203	1951	Крысиный (15)	25	25	47	0	44	—	+	++
363	1959	Сурчиный (14)	25	25	40	0	43	—	+	++
380	1959	Песчаночий (16)	25	25	68	0	63	—	+	++

1 - Strain
2 - Year isolated
3 - Focus
4 - Virulence, microbial cells
5 - guinea pigs
6 - white mice
7 - Of 100 microbial cells there grow on media
8 - with 5% blood
9 - Higuchi
10 - Fermentation
11 - rhamnose
12 - glycerin
13 - Lysis by plague bacteriophage
14 - Marmot
15 - Rat
16 - Gerbil

The work was done only with the generation grown at 28°. Subcultures were transferred from the original culture of each strain to several test tubes with slant agar prepared from a hydrolyzate of blood clots. These basic subcultures were incubated at 28° for 20 hours, sealed up, and stored for the time of the experiment (3 years) in a refrigerator. There were no transfers of the basic subcultures.

Transfers were made in each experiment from the basic subcultures to a fresh medium and isolated colonies were obtained from the renewed culture. These colonies were poured off into test tubes with slant agar and after the cultures were incubated for 20 hours, they were tested for virulence on white mice, from 5 to 10 animals per culture obtained from each colony. The mice

were inoculated with 10,000 microbes. The dose was large because we wished to level out in part the individual fluctuations in susceptibility among the mice and at the same time make the differences in virulence of the strains more significant. According to our data (Malinina and Knyazeva, manuscript), highly virulent strains exhibit slight differences in virulence when small doses are used. When they are increased to more than 10,000 bacterial cells, these differences for white mice disappear. However, if a difference in virulence does appear with large doses, it is even more significant.

The cultures was inoculated subcutaneously in a volume of 0.1 ml in the right leg. A total of 2500 animals were used in the experiment. Virulence was studied in 75 colonies of each strain. Since the results were approximately the same for each colony, we thought we could simplify the table and present the data for only 10 colonies (Table 2, which reflects the materials of three years' observations on changes in the number of virulent individuals in standard strains isolated from gerbil, marmot, and rat foci).

It is evident from Table 2 that the cell composition of virulent strains of the plague microbe isolated from various carriers, which kill experimental animals in a dose of 25 microbes in the general population, changes in various ways the longer the strains are stored on nutrient media.

The cell composition of strain 293 of rat origin, despite prolonged storage on nutrient media, was fairly stable. During the first two years' observation on the population, we noted that it consisted mainly of virulent individuals. Only in the third year did we detect one colony (out of 10) from which a culture proved to be safe for white mice in the dose tested. All the animals infected with 10,000 microbes of this subculture survived. Moreover, further testing of the immunogenic properties of the subculture showed that the aforementioned dose does not protect animals from reinfection. Consequently, this subculture, in addition to change in virulence, also lost its immunogenic properties. Study of the fermenting properties of strain 293 revealed that its population included isolated colonies capable of utilizing rhamnose at later periods.

A similar pattern was also noted in the experiment with freshly isolated strain 363 of marmot origin. Throughout the observation period we were never able to isolate a subculture which in a dose of 10,000 microbes would not kill all the experimental animals. The percentage of virulent individuals in the strain was not only high, but it remained the same during all the years of observation. The high virulence of the strain is also indicated by the results of bacteriological control of the surviving animals. In the animals sacrificed 18-20 days after inoculation with strain 363, the microbes

were generally isolated not only from the injection site, but from the viscera as well. Consequently, the rate of "take" and infectivity even in cells with weakened virulence was high. Moreover, all the subcultures from strain 363 survived and created fairly strong immunity in the animals. None of the mice died when they were reinfected.

Table 2

Virulence of Isolated Colonies of Plague Strains of Different Origin

(1) Год наблюдения	(2) Количество колоний из 10, от которых животные:						(8) Из 10 колоний ферментируют		(11) Средняя скорость редукции метиленовой синьки, мин.
	(3) все пали		(4) частично выжили		(5) все выжили		(9) глицерин	(10) рамнозу	
	(6) число колоний	(7) число животных	(6) число колоний	(7) число животных	(6) число колоний	(7) число животных			
	(12) Штамм 293								
1961	9	90	1	9/1	—	—	—	—	119
1962	10	50	—	—	—	—	—	—	131
1963	9	45	—	—	1	5	—	1	174
	(13) Штамм 363								
1961	9	90	1	9/1	—	—	10	—	105
1962	6	30	4	16/4	—	—	10	2	140
1963	6	30	4	16/4	—	—	8	—	150
	(14) Штамм 380								
1961	7	70	3	25/5	—	—	9	—	190
1962	5	25	3	10/5	2	10	10	—	199
1963	5	25	1	1/4	4	20	10	1	225

Note. In the numerator - dead mice; in the denominator - surviving mice.

1 - Year of observation
2 - Number of colonies (10), from which the animals
3 - all died
4 - some survived
5 - all survived
6 - number of colonies
7 - number of animals
8 - Of 10 colonies that ferment
9 - glycerin
10 - rhamnose
11 - Average rate of reduction with methylene blue, min

In strain 363, like the oceanic strain (293), study of the cell composition revealed occasional colonies that ferment l rhamnose. Howeve., the most interesting thing about this strain, which usually ferments glycerin, was our ability to isolate two variants which proved to be incapable of utilizing it. Moreover, this property was stable. Continued subculturing failed to restore the lost characteristic.

The freshly isolated gerbil strain (380) presented a somewhat different and fairly clear-cut picture of changes in cell composition. Storage on nutrient media without transfer resulted in the cell composition becoming extremely unstable. Although the experimental conditions were the same for all the strains, only in the case of the gerbil strain were we able to isolate a subculture with distinctly less virulence after a year of observation. In a dose of 10,000 microbes it failed to kill the experimental animals. After 3 years of storage on a nutrient medium without transfer, 5 of 10 colonies taken from the general population were harmless to white mice. In addition, there was a marked change in the rate of "take" of a culture from these colonies. Bacteriological analysis of the sacrificed animals showed that microbes were isolated only from occasional animals and solely from the inoculation site.

In testing the immunogenic properties of subcultures from individual colonies in strain 380, we found in almost half the cases colonies that did not protect white mice after reinfection. While studying the fermenting ability of individual colonies, we isolated one that did not ferment glycerin. However, this property in the given culture, unlike the variants isolated from strain 363, was unstable. Subsequent passage of the glycerin-negative culture through white mice resulted in loss of this characteristic. We also found rhamnose-positive variants in strain 380. Regarding the frequency of appearance of rhamnose-positive colonies in the strains whose general population usually does not ferment rhamnose, we found them quite often in the gerbil strain. For example, we were able to isolate more rhamnose-positive colonies from the 75 studied in the gerbil strain (10) than we did in the marmot (4) or rat (2) strains. However, we could not detect in individual colonies of all the strains a close relationship between a decrease in virulence and development of the capacity to utilize rhamnose. The ability to utilize rhamnose was found both in colonies with highly virulent strains and in colonies with low virulence.

Thus, of the three highly virulent strains that we isolated from various carriers, strain 380 of gerbil origin was the least stable in cell composition. On the other hand, the strains isolated from rat and marmot foci were fairly homogeneous and stable in this respect. This was true not only of the freshly isolated ones, i.e., stored only briefly on nutrient media (marmot origin), but also of the strains stored on nutrient media about 15 years (Table 3).

Table 3

Comparison of the Virulence of Isolated Colonies in Strains of Marmot Origin (Freshly Isolated and Stored for a Long Time on Media)

(1) Штамм	(2) Число колоний из 10, от которых животные, зараженные в дозе											
	(3) 10 тыс. м. т.				(4) 1 тыс. м. т.				(5) 100 м. т.			
	(6) пали		(7) частично выжили		(6) пали		(7) частично выжили		(6) пали		(7) частично выжили	
	(8) число колоний	(9) число животных	(8) число колоний	(9) число животных	(8) число колоний	(9) число животных	(8) число колоний	(9) число животных	(8) число колоний	(9) число животных	(8) число колоний	(9) число животных
231	8	80	2	18/2	10	100	—	—	9	90	1	9/1
363	9	90	1	9/1	9	90	1	90	9	90	1	9/1

Note. The same as that in Table 2.

1 - Strain
2 - Number of colonies (10), from which the animals inoculated with a dose of
3 - 10,000 microbial cells
4 - 1000 microbial cells
5 - 100 microbial cells
6 - died
7 - partly survived
8 - number of colonies
9 - number of animals

It is evident from Table 3 that the virulence of individual cells was virtually the same in strains 231 and 363. Both strains consisted of highly virulent individuals that killed almost all the animals in doses of 10,000, 1000, and 100 microbes.

The marked heterogeneity of the cell composition of the gerbil strain as compared with the marmot and rat strains may be explained, according to G. N. Lenskaya (1959), by the fact that rodents play a major role in formation of the strain in the natural focus and when highly virulent microbes are transferred from the natural reservoir to a synthetic nutrient medium, it accelerates only to a certain degree the process of variability that started in the organism of the carrier, the gerbil in particular. The following should be borne in mind when correlating the virulence of individual colonies in a strain and their morphology, ability to ferment glycerin and rhamnose, and reducing activity. A careful study of the cell composition of four strains whose general population is incapable of utilizing rhamnose invariably reveals occasional colonies

which ferment this carbohydrate. In strain 380, the cells that ferment rhamnose are much more numerous than those in strains 231 and 363 and especially 293. Worth noting is the late start of fermentation of rhamnose - 9th to 12th days.

Our observations on the fermentation of glycerin are of interest. From the population of two glycerin-positive strains, we isolated two colonies from strain 363 and one from strain 380, which were incapable of utilizing glycerin. This property was unstable in strain 380. The virulence of the glycerin-negative variants was the same as in the original strains.

The reducing activity of the highly virulent strains studied decreased in direct proportion to the length of time they were stored on nutrient media without subculturing.

Conclusions

1. The microbial population of highly virulent standard strains of the plague microbe are heterogeneous in cell composition. Individual colonies of the same strain differ from one another in virulence, immunogenicity, and fermenting activity. The most stable over a period of years and most alike in virulent properties are the rat and marmot strains, both freshly isolated and stored for a long time on nutrient media. A freshly isolated gerbil strain is less stable and uniform in cell composition. When stored on nutrient media without subculturing, the number of individuals with marked decrease in virulence grows from year to year.

2. Virulent plague strains belonging to the continental variety contained colonies incapable of utilizing glycerin. Two glycerin-negative variants were isolated from strain 363; one, from strain 380. The glycerin-negative variant in the gerbil strain proved to be unstable.

3. In the highly virulent strains, no close relationship was noted between the capacity of the colonies to ferment rhamnose or glycerin and virulence. Colonies that fermented rhamnose but not glycerin were highly virulent.

4. The stability of all the basic characteristics, chiefly virulence, in the standard plague strains is largely determined by the nature of the strain, i.e., by the source from which it is isolated.

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